Description

METHOD FOR SEPARATION OF COMPOUNDS
USING CYCLING DENATURING CONDITIONS

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority from U.S.
provisional application no. 60/430,169, filed December 2,
2002.

TECHNICAL FIELD

The present invention relates generally to
separation and analysis of compounds and more
specifically to means of separating compounds based on
their secondary structure under partial or fully
denaturing conditions.

BACKGROUND OF THE INVENTION

Analysis of DNA variations (mutations or
polymorphisms) is central to many applications in
genetics, genomics as well as in clinical research and
diagnostics. One commonly used approach is based on
separation of PCR amplified fragment of a target sequence
under partial denaturing conditions. The partial
denaturation is usually achieved by a chemical denaturant
or raising the temperature.

Maintaining an accurate optimum temperature at
which mutants in the studied target sequence possesses
different conformation from the non-mutated (wildtype)
sequence over the duration of electrophoretic or
chromatographic separation is generally difficult. A
more common approach is to use a temperature gradient
during which sooner or later the separated fragments
reach their optimum temperature, provided that the
temperature range of such gradient is sufficiently wide
(Schell et at. 1999). Applying temperature gradients

became recently popular in multicapillary electrophoresis systems (Li et al. 2002). With temperature gradients, the reproducibility among different capillaries in an array is better compared to constant temperature control

5   approach. However one problem still remains. The period of time during which the fragment is subjected to its melting optimum depends of the gradient slope i.e. rate at which the temperature is changed. Prior to analysis of an unknown sample it is essential to optimize the

10   temperature settings with respect to the temperature range and gradient slope. Also in some multi-channel systems, it is difficult to control a single-sweep temperature gradient profile reproducibly in all channels at once. As a result the resolution as well as channel-

15   to-channel reproducibility is often not optimal. Finally the overall sample throughput of a single-sweep gradient system is limited due to the relatively long time duration required for the gradient to be completed over the entire course of the separation.

20   It is an object of this invention to provide a method for separation of compounds (such as DNA molecules) under partially or fully denaturing conditions.

          It is a further object of the invention to

25   provide a method not requiring complex optimization of separation parameters such as the denaturant gradient slope.

          It is a further object of the invention to provide a method applicable to detection of DNA mutations

30   and polymorphisms using multiple-injection technology for increased sample throughput.

          It is a further object of the invention to provide a method applicable to multi-channel separation instruments without a need of complex changes in

35   instrumentation design.

SUMMARY OF THE INVENTION

The above objects have been achieved through application of multiple cycles of temperature gradient during the course of separation. During each of the

5   cycles the temperature is changed from a starting temperature point to the ending temperature point, at some point reaching partial or complete melting temperature (Tm) of each of the individual components within the sample mixture. Melting in this context means

10   separation of double stranded DNA into single strands. The periodical exposition of the components to their melting temperatures aids in their separation by means of electrophoresis, chromatography or other processes applied in the system. The revolving optimum separation

15   conditions allow for application of multiple-injection technology where different sample mixtures may be periodically injected in phase with the repeating cycles. The separation can be performed in a single column (e.g. single capillary tube) or a micro-fabricated channel

20   (e.g. channels in a substrate or chip) or in an array of columns or micro-fabricated channels. The main area of applicability is in high-throughput discovery, detection and screening of genetic variations (DNA mutations and polymorphisms).

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a graph of temperature over time in constant temperature separations.

Figure 1B is a schematic of a capillary tube

30   during a single injection, constant temperature separation.

Figure 2A is a graph of a single-sweep temperature gradient.

Figure 2B is a schematic of a capillary tube

35   during a single injection during a typical temperature-gradient capillary electrophoresis (TGCE).

Figure 4 is a graph of the temperature profile inside MEGABACE 1000® capillary array instrument during a cycling temperature gradient experiment.

Figure 5 shows a readout of optically detected signals during analysis of a multiple-injection separation of DNA mutants using cycling temperature gradient conditions.

DETAILED DESCRIPTION OF THE INVENTION

In the described method a new approach to separation of compounds from mixtures based their partial or complete denaturation is presented. In one embodiment it utilizes a series of temperature gradient cycles to achieve revolving optimum conditions to separate individual compounds from a sample mixture. The periodical application of the optimum temperature conditions aids separation in which multiple samples are injected in the course of a single experiment by means for multiple-injection technology, such as U.S. Patent No. 6,156,178 hereby expressly incorporated by reference herein.

With reference to Figure 1B, during a classical capillary electrophoretic separation a sample 10 is injected onto a separation channel 20 (e.g. capillary) and separated at a constant temperature. The applied separation temperature needs to be previously determined to be optimum for ideal resolution of compounds within the sample mixture (as in U.S. Patent No. 5,633,129 to Karger et al. hereby incorporated by reference herein). Maintaining accurate constant temperature is relatively difficult especially in multichannel systems, where a plurality of samples are injected and separated in parallel. There the overall heat dissipation usually results in a higher temperature in channels closer to the array center and lower temperature in the (better cooled) channels on its sides. Even relatively small differences

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(< 0.5 C) might result in significant changes in separation patterns if the same sample is analyzed in different channels. Temperature over time is graphically illustrated in Figure 1A.

5   One partial solution of the non-uniform temperature distribution problem is by application of a single-sweep temporal temperature gradient. With this approach each channel undergoes a range of changing conditions, but the overall impact of the partial

10 denaturation in all channels is roughly equilibrated. Yet, with single-sweep temperature gradient (as shown in graph 2A) it is still necessary to carefully optimize the temperature gradient slope to match the duration of sample migration for each of the sample compounds to be

15 analyzed. As in Figure 1B, Figure 2B indicates a single sample 10 is separated in capillary 20.

         Figure 3A shows a graph illustrating the proposed improvement of the single-sweep temperature gradient technique by applying a series of temperature

20 cycles. With such cycling temperature gradients, there is no need for matching the gradient slope to the duration of the electrophoretic migration since during each cycle, channels are repeatedly subjected to optimum separation temperatures. In addition to the overall

25 simplification of the technique; the use of periodical cycling gradient further enables a significant increase in sample throughput by application of multiple-injection technique as illustrated in Figure 3B. In Figure 3B samples 10a-10d are injected into capillary 20 at

30 intervals. In a multiple-injection experiment, samples are serially injected onto a separation channel (or an array of channels) in periodical time intervals (see Mansfield et al. U.S. Patent No. 6,156,178). The main advantage of this approach is increase in a number of

35 samples analyzed per experiment. Applying multiple injection method is not possible with single-sweep

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temperature gradients, since the samples eluting at the early stage of the experiment would be subjected to different temperature range compared to the ones injected later. With the temperature gradient cycling each of the serially injected samples undergoes similar number of cycles before reaching the detector, thus is subjected to equivalent separation conditions. As illustrated in Figure 3A, the cycles are uniform temperature cycles. However, either or both of the temperature range or duration of the cycles may be altered. It is still preferred that in the interval between subsequent injections the temperature cycles be identical (e.g. if the pattern is one long and two short cycles from high to low temperature, this pattern be repeated between each sample injection.

Figure 4 shows a temperature profile recorded during a cycling temperature gradient experiment using MEGABACE 1000® capillary array instrument. The cycling gradient range was set from 54°C to 46°C with a cycling frequency of 1 cycle per 10 minutes.

Figure 5 shows a practical example of application of cycling temperature gradient approach. Four different DNA samples (k-ras exon 1 PCR amplicons from colorectal tumor samples) were serially injected onto a single capillary and analyzed using cycling gradient conditions with cycling temperature range from 52°C to 50°C and cycle rate of 2 minutes per cycle.

Those of skill in the art would recognize that the disclosed temperature cycling during electrophoresis could be used on single capillary systems, capillary array systems, or capillary (microchannel) chips. Any of a number of available systems could be used for electrophoretically separating and detecting samples. Detectors include fluorescence detectors, mass spectrometry detectors and others. If a commercially available system is used, the system can be instructed to

automatically cycle between a high and low temperature. If multiple samples are injected, a sample is injected followed by a separation interval, followed by a second injection, followed by a second separation interval.

5   This process is repeated until all samples are loaded into the capillary. All of the samples are then continuously detected. It is preferred that during the time between injections the sample be cycled between high and low temperature settings at least 2 times.